AD-A284 [redacted]

ENTATION PAGE

Form Approved
OMB No. 0704-0188

mated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, d reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this s burden, to Washington Headquarters Services, Directorate for information Operations and Reports, 1215 Jefferson e Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	7/28/94	~~Interim~~ Annual 7/1/93 - 7/31/94

4. TITLE AND SUBTITLE

Cellular Analysis of Circadian Rhythmicity in Cultured SCN Neurons

5. FUNDING NUMBERS

G F49620-93-1-0434

2312/CS

61102F

6. AUTHOR(S)

Steven M. Reppert & David K. Welsh

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts General Hosp
Fruit Street
Boston, MA 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Dr. Genevieve Haddad
AFOSR/NL
110 Duncan Ave Suite B115
Bolling AFB DC 20332-0001

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

AFOSR-TR- 94 0495

11. SUPPLEMENTARY NOTES

DTIC ELECTE SEP 07 1994 F

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release;
distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Circadian rhythms are generated by brain cells located in the suprachiasmatic nuclei (SCN) of the mammalian hypothalamus, but it is not clear how individual cells contribute to the operation of the circadian clock. SCN neurons dissociated from newborn rat SCN were characterized by immunocytochemistry and by patch recording of spontaneous action potentials and synaptic currents. Inhibitory synaptic interactions were prevalent among neurons, increasing progressively with time in culture. Evidence was found for presence of gap junctions between glial cells but not between neurons. To assess circadian rhythmicity, long-term multielectrode recordings of spontaneous action potentials were obtained from neurons cultured for 1-6 wks on glass plates containing a flat array of 61 microelectrodes. Firing rates of some but not all individual neurons exhibited clear circadian rhythms with periods of nearly 24 hrs. Within a culture, cells expressing circadian rhythms showed no short-term firing synchrony. The phase of the circadian rhythm varied among cultures and, in most cases, among different cells within the same culture. With the ability to record circadian rhythms from individual SCN neurons, experimental analysis can now proceed to detailed study of circadian pacemaker neurons and their interactions.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Circadian Rhythms DTIC QUALITY INSPECTED 3	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

NSN 7540-01-280-5500

Standard Form 298 (Rev 2-89)
Prescribed by ANSI Std Z39-18
298-102

AUG 1994

FROM: David K. Welsh, Steven M. Reppert
Laboratory of Developmental Chronobiology, Jackson 1226
Massachusetts General Hospital
Boston, MA 02114
phone (617) 726-8450, fax (617) 726-1694

94-28982

TO: Marily J. McKee
AFOSR/NL
110 Duncan Avenue, Suite B115
Bolling AFB, DC 20332-0001
phone (202) 767-4949, fax (202) 404-7951

SUBJ: Technical Report, due 7/31/94

Grant Number: F49620-93-1-0434DEF. **Topic Title**: Circadian Rhythmicity in Cultured SCN. **Performance Site**: Laboratory of Developmental Chronobiology, Jackson 1226, Massachusetts General Hospital, Boston, MA 02114.

(A) Objectives:

Circadian rhythms in mammals are generated by a biological clock located within the hypothalamus, in the suprachiasmatic nuclei (SCN). This conclusion has been established by a variety of experimental approaches, including lesions of SCN, transplantation of fetal SCN into SCN-lesioned hosts, and expression of circadian rhythms by isolated SCN tissue in vitro. The fundamental question of how the circadian clock is organized at a cellular level, however, has proven difficult to approach experimentally. This has been a serious obstacle to further work on the cellular and molecular mechanisms of circadian rhythmicity in mammals. We aim to address fundamental questions about the cellular organization of the circadian clock within the SCN by taking advantage of a novel in vitro assay of circadian rhythmicity, whereby circadian variation in the rate of spontaneous action potentials can be recorded over a period of weeks from multiple neurons in cultures of cells dissociated from neonatal rat SCN. Our specific objectives are to:

1. Analyze the development in vitro of circadian oscillations in cultures of dissociated SCN cells. Other groups have shown that SCN cells cultured together in the same dish can express a coordinated circadian rhythm. We will investigate how this circadian rhythmicity emerges at a cellular level by recording spontaneous action potentials from individual neurons at frequent intervals during the first 4 weeks in culture. We will determine (a) when circadian rhythmicity is first evident in individual neurons, (b) to what extent coordinated circadian rhythmicity is achieved among different neurons recorded in the same culture, and (c) whether synchronization of circadian oscillations among different cells varies with the distance between cells in the culture dish or the length of time since the cells were initially dissociated.

2. Evaluate the importance of specific forms of intercellular communication for expression of circadian rhythmicity in vitro. Intercellular communication of some type is surely necessary for expression of a uniform circadian rhythm by SCN cells in a culture, and may be required for expression of circadian rhythms in any individual cell. The possible roles of chemical synapses, gap junctions, glial interactions, and humoral communication will be evaluated. We will (a) selectively disrupt each of these four possible modes of intercellular communication in SCN cultures, and assess the effect on circadian rhythmicity. We will also (b) measure quantitatively the prevalence of chemical synapses and gap junctions among SCN cells at 1, 2, and 4 days, and 1, 2, and 3 weeks in culture, and then attempt to correlate these findings with the time course of development of circadian function in companion cultures.

DTIC QUALITY INSPECTED 8

94 9 06 056

3. Characterize single SCN neurons expressing circadian rhythmicity. We will (a) characterize neurons expressing circadian rhythms by morphology, neuropeptide content, and short-term firing patterns. We will also (b) quantitatively assess the expression of voltage-dependent ionic currents in these neurons by whole cell patch clamp recording. Neurons will be studied either at the peak or the trough of their circadian rhythms of firing rate, to determine whether circadian modulations of specific ionic currents underlie the circadian variation of spontaneous firing rate.

(B) Status:

1. Analyze the development in vitro of circadian oscillations in cultures of dissociated SCN cells. SCN cultures were validated by immunocytochemical staining for GABA, vasopressin, VIP, somatostatin, and oxytocin. Spontaneous action potentials were recorded from individual SCN neurons using both cell attached patch and multielectrode plate techniques. Multielectrode recordings lasting 3 days or more have reproducibly detected clear circadian rhythms in rate of spontaneous firing in approximately half of neurons recorded in SCN cultures. Typically, these cells were virtually silent for many hours, before firing rate abruptly increased to near maximal levels at a consistent time each day. The periods of these rhythms were very close to 24 hrs. No such rhythms were detected in cultures of hippocampal cells. Circadian firing rhythms have been recorded from SCN neurons as early as 1 week in culture, and as late as 6 weeks in culture. In some cases, the same SCN cells could be recorded repeatedly over periods of several weeks, revealing long-term stability of circadian oscillations in individual cells. In simultaneous recordings of multiple SCN neurons in the same culture, the cells were usually found to express circadian rhythms of different phases. With additional experiments, we will determine whether this desynchrony is affected by distance between cells or time in culture.

2. Evaluate the importance of specific forms of intercellular communication for expression of circadian rhythmicity *in vitro*. The prevalence of synaptic interactions among SCN cells was assessed at 1, 2, and 4 days, and 1, 2, and 3 weeks in culture. In whole cell patch recordings, the proportion of cells exhibiting spontaneous synaptic activity increased rapidly from ~15% at 1 day to 80-90% by 4 days in culture. The rate of synaptic currents, however, continued to increase progressively with time in culture, up to 2 weeks. This was partly due to an increase in number of synapses, as demonstrated by immunostaining for the synaptic vesicle protein SV2, and partly due to an increase in rate of spontaneous firing, as demonstrated by cell attached patch recording. The great majority of these synaptic currents were inhibitory GABA-A currents, blocked by bicuculline and reversing near the equilibrium potential for chloride. Despite abundant evidence of synaptic interactions, however, SCN cells in the same culture often expressed circadian firing rhythms of different phases, suggesting that conventional synaptic interactions are not sufficient to synchronize circadian oscillators in SCN cultures. Conversely, even cells expressing synchronized circadian rhythms showed no short-term firing correlations, suggesting that strong synaptic coupling is not necessary to synchronize SCN circadian oscillators.

The presence of gap junctions was assessed by immunostaining for specific gap junction (connexin) molecules, by filling of SCN neurons and astrocytes with Neurobiotin, a tracer compound known to traverse gap junctions, and by simultaneous recording of action potentials from multiple SCN neurons. No evidence was found at any culture age for gap junctions between neurons. Between astrocytes, however, Neurobiotin coupling and punctate staining for connexin-43 were observed by 1-2 weeks in culture, suggesting the presence of gap junctional coupling.

In future experiments, we will attempt to disrupt various forms of intercellular communication in SCN cultures, and assess the effect on circadian rhythmicity. In consideration of our evidence of desynchrony among circadian firing rhythms of different SCN cells in the same culture, we will also attempt to induce synchrony by timed appplications of melatonin or other putative diffusible intercellular messengers, such as NO or CO.

3. Characterize single SCN neurons expressing circadian rhythmicity. Preliminary *data suggest that SCN neurons expressing circadian rhythms may be distinguishable from other* cells lacking such rhythms by the regularity of their short-term firing patterns. In future experiments, cells expressing circadian rhythms in multielectrode recordings will be identified unambiguously by simultaneous cell attached patch recording, and then studied in detail by whole cell recording or immunostaining for vasopressin and VIP. In later experiments, neurons will be studied either at the peak or the trough of their circadian firing rhythms, to determine whether circadian modul[illegible] of specific ionic currents underlie the circadian variation of firing rate.

(C) Publications:

1. Welsh DK, Logothetis DE, Reppert SM. Spontaneous electrical activity in neurons cultured from rat suprachiasmatic nucleus (#176). *Soc Res Biol Rhythms Abstr* 4:120, 1994.

2. Welsh DK, Logothetis DE, Reppert SM. Circadian firing rhythms of neurons dissociated from rat SCN. *Soc Neurosci Abstr*, submitted, 1994.

3. Welsh DK, Logothetis DE, Meister M, Reppert SM, "Circadian firing rhythms of individual neurons dissociated from rat suprachiasmatic nuclei", in preparation for submission to *Neuron*, 1994.

(D) Personnel:

1. Steven M. Reppert, Professor of Pediatrics, Children's Service, Massachusetts General Hospital, and Program in Neuroscience, Harvard Medical School

2. David K. Welsh, M.D./Ph.D. candidate, Program in Neuroscience, Harvard Medical School

(E) Interactions:

1. poster presentation, "Synaptic and nonsynaptic communication among cells cultured from rat suprachiasmatic nucleus", Gordon Research Conference on Chronobiology, New London, NH, 1993

2. oral and poster presentations, "Cultured SCN neurons express circadian rhythms in firing rate", Children's Service Annual Research Symposium, Massachusetts General Hospital, 4/94

3. poster presentation, "Circadian firing rhythms of individual neurons dissociated from rat SCN", Society for Research on Biological Rhythms, Jacksonville, FL, 5/94

4. oral presentation, "Single cell SCN recordings", Biological Clockwatchers Society (Harvard Medical School), Boston, MA, 5/94

(F) Inventions and Patents: none.

(G) Other Statements: none.

Accesion For
NTIS CRA&I ☒
DTIC TAB ☐
Unannounced ☐
Justification .....
By .....
Distribution /
Availability Codes
Dist | Avail and / or Special
A-1